IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

DENNY et al — Atty. Ref.: 5011-6

Appl. No. 10/590,796 — TC/A.U. 1624

Filed: March 1, 2005 — Examiner: Balasubramanian, V

For: NOVEL 1,2,4-BENZOTRIAZINE-1,4-DIOXIDES

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION

I, William R. Wilson, hereby declare and state that:

1. I am a co-applicant in the above application.

2. The following experiments have been performed under my direct supervision and control.

3. **Measurement of maximum tolerated doses in CD-1 nude mice.** Toxicity of the test articles tirapazamine (3 amino-1,2,4- benzotriazine 1,4-dioxide) and SN 29751 (3-Ethyl-6-[3-(4-morpholinyl)propoxy]-1,2,4-benzotriazine 1,4-dioxide, hydrochloride salt) was evaluated in male CD-1 homozygous nude mice bred in the Animal Resources Unit, The University of Auckland. Animals were approximately 6 weeks of age when used, and were housed in groups of up to 6 in a temperature-controlled room (22 ± 2°C) with a 12-hour light/dark cycle and were fed *ad libitum* water and a standard rodent diet (Harlan Teklad diet 2018i). All animals were uniquely identifiable by ear tag number. The compounds were formulated in saline, filtered through a 0.22 µm filter and concentrations of the stock solutions determined by spectrophotometry using an

WRW

extinction coefficient of 39616 $M^{-1}cm^{1}$ at 266 nm for tirapazamine, and 11606 $M^{-1}cm^{1}$ at 369 nm for SN 29751. Stock solutions were held at room temperature in vials wrapped in foil for up to 8 hr. Animals were dosed intraperitoneally (IP) using two different schedules: single dose and bi-daily for four consecutive days (9 am and 3 pm daily; BID1-4). Initially, each compound was administered to three mice at a dose expected to be close to the MTD based on in vitro cytotoxicity. Dose levels were set using a fixed 8-step scale ($1/8^{th}$ log_{10} increments, at values of 1, 1.33, 1.78, 2.37 etc), using molar units (µmol/kg), which were then converted to mass units (mg/kg) using the MW of TPZ (178) or SN 29751 (371). Mice were observed for clinical signs of toxicity twice on the first day and observed and weighed daily for the first five days then approximately three times a week until 28 days after dosing. Animals were culled if they lost greater than 15% of their initial weight or demonstrated severe clinical signs such as convulsions, diarrhoea, immobility or lethargy persisting for more than 24 hr. Based on this initial dose level, further animals were dosed at a higher or lower dose or at the same dose level as required to determine the maximum tolerated dose (MTD), which was defined as the highest dose that did not cause lethality or unacceptable morbidity or unacceptable weight loss (more than 15% of pre-treatment body weight) in any of at least six animals.

4. **Measurement of tumour cell killing in human tumour xenografts**. The ability of the test articles to kill hypoxic tumour cells was assessed by combining with ionising radiation (to kill aerobic tumour cells) and removing the tumours to enumerate clonogenic (colony forming) survivors in culture, which is a well-validated assay for hypoxic cell killing in tumours. Human tumour cell lines (SiHa cervical carcinoma, HT29 colon carcinoma, H460 non small cell lung cancer and H1299 non small cell lung cancer) were passaged in culture and grown as xenografts by subcutaneous inoculation of 10^{7} cells in CD-1 nude mice. For single dose studies the inoculation site was on the right flank, while for multidose studies (requiring targeting of radiation to the tumour volume) tumours were inoculated 1.5 cm from the base of the tail on the midline under Avertin anaesthesia. Tumour growth was monitored by calliper measurement until tumours reached a mean diameter of 9-11 mm (approximately 350 mg) at which time they were randomised to treatment groups. The test articles were formulated for dosing as in the toxicity studies above.

   The test articles were dosed IP at a range of doses as indicated in the Figures, either as single agents or at various times before and after gamma irradiation using a cobalt-60 source (Eldorado G). Control animals received an equivalent volume of saline. Radiation was delivered either whole body using unrestrained mice (for single dose studies) or locally to the tumour-bearing region in restrained mice for fractionated dosing studies

by twice-daily dosing at 9 am and 3 pm daily for four consecutive days. Radiation doses were 15 Gy or 20 Gy for single dose studies and 2.0 or 2.5 Gy/dose for fractionated dosing studies.

18 hr after completion of treatment, mice were killed by cervical dislocation, tumours removed by sterile dissection, minced using curved scissors and crossed scalpels, and up to 500 mg transferred to pre-tared sterile 14 ml tubes containing magnetic spin bars. An enzyme cocktail comprising Pronase (Sigma P-5147, 2.5mg/ml), collagenase (Sigma C-5138, 1mg/ml) and DNAase I (Sigma DN-25, 0.2mg/ml) in culture medium (alphaMEM with 10% fetal calf serum; "medium") was added at a rate of 1ml/50mg tumour. Samples were incubated with magnetic stirring at 37°C for 30 min (SiHa, H460) or 45 min (HT29, H1299). A 1 ml aliquot was then diluted into fresh medium, centrifuged, resuspended in 10 ml fresh medium, and cell density determined with an electronic particle counter (Beckman Coulter Electronics model Z2). Samples were diluted to 10^5 cells/ml, and 6-fold serial dilutions made down to 460 cells/ml. Aliquots (1 ml) of each dilution were plated in triplicate in P60 dishes containing 4 ml medium. Plates were incubated in 5% CO_2 incubators at 37°C for 10 days (H1299, H460) or 14 days (HT29, SiHa) then fixed and stained with 1% methylene blue in 50% EtOH. Colonies containing >50 cells were scored as clonogenic survivors, and the number of clonogenic survivors per gram tumour tissue was calculated.

Log cell kill by the drug alone or radiation alone was calculated as $\log_{10}$(clonogens/g for control mice) minus $\log_{10}$(clonogens/g for treated mice). Log kill additional to radiation was calculated as $\log_{10}$(clonogens/g for radiation only) minus $\log_{10}$ (clonogens/g for the combined treatment). Statistical analysis was conducted using ANOVA with Dunnett's test (SigmaStat v3.5) to separately evaluate the significance of drug effects in the drug alone versus control, and drug plus radiation versus radiation alone.

5. **The results are shown** in the attached Figures 1 to 6. These figures are described below:

Figure 1. Time course of interaction with single dose radiation, SiHa tumours. Tirapazamine (TPZ) or SN 29751 was administered IP to mice with subcutaneous SiHa tumours at various times before or after a single dose of ionising radiation (15 Gy, whole body). Tumours were excised 18 hr after the end of treatment, and the number of surviving tumour clonogens determined in culture. Values are mean ± SEM for groups of 4-5 mice. The log cell kill for radiation only was 1.87 ± 0.10. All values for drug plus radiation were statistically significantly different from radiation alone ($p<0.05$) by ANOVA/Dunnett's.

Figure 2. Comparison of TPZ (23.7 mg/kg) and SN 29751 (278 mg/kg) against hypoxic cells in three human tumour xenograft models. Compounds were administered IP to mice with subcutaneous tumours 5 min after a single dose of radiation (HT29, 15 Gy; SiHa and H460, 20 Gy). Tumours were excised 18 hr after the end of treatment, and the number of surviving tumour clonogens determined in culture. Values are mean ± SEM for groups of 4-5 mice. The log cell kill for radiation only was 1.89 ± 0.65 for HT29, 1.87 ± 0.10 for SiHa and 1.59 ± 0.10 for H460. The values for SiHa are redrawn from Figure 1. All values for drug plus radiation were statistically significantly different from radiation alone ($p<0.05$) by ANOVA/Dunnett's.

Figure 3. Comparison of TPZ and SN 29751 in combination with fractionated radiation in two human tumour xenograft models. Compounds were administered IP 30 min before each dose of radiation using a bi-daily schedule for 4 consecutive days (BID 1-4, 8 x 2.5 Gy). Tumours were excised 18 hr after the end of the final treatment, and the number of surviving tumour clonogens determined in culture. Values are mean ± SEM for groups of 5 mice. The log cell kill for radiation only was 1.57 ± 0.09 for H460 in expt 1, 1.36 ± 0.19 for H460 in expt 2, and 1.76 ± 0.12 for H1299. Statistically significant differences by ANOVA/Dunnett's are shown in the figure.

Figure 4. Time course of interaction of TPZ or SN 29751 with fractionated radiation against SiHa tumours. Compounds were administered IP at the indicated times before or after each dose of radiation using a bi-daily schedule for 4 consecutive days (BID 1-4, 8 x 2 Gy). Tumours were excised 18 hr after the end of the final treatment, and the number of surviving tumour clonogens determined in culture. Values are mean ± SEM for groups of 5-6 mice. The log cell kill for radiation only was 1.67 ± 0.04, while TPZ alone gave a log cell kill of 0.19 ± 0.23 ($p = 0.46$) and SN 29751 alone gave a log cell kill of 0.56 ± 0.22 ($p = 0.07$). Activity of SN 29751 with radiation was significantly greater ($p<0.05$) than for radiation alone and for radiation plus tirapazamine at each timpoint by ANOVA/Dunnett's.

Figure 5. Dose response for interaction of TPZ or SN 29751 with fractionated radiation against SiHa tumours. Compounds were administered IP 30 min before each dose of radiation using a bi-daily schedule for 4 consecutive days (BID 1-4, 8 x 2 Gy). Tumours were excised 18 hr after the end of the final treatment, and the number of surviving tumour clonogens determined in culture. Values are mean ± SEM for groups of 6 mice. The activity of both TPZ and SN 29751 with radiation was significantly greater ($p<0.05$) than for radiation alone by ANOVA/Dunnett's at all doses.

Figure 6. Dose response for interaction of TPZ or SN 29751 with fractionated radiation against H460 tumours. Compounds were administered IP 30 min before each dose of

radiation using a bi-daily schedule for 4 consecutive days (BID 1-4, 8 x 2.5 Gy). Tumours were excised 18 hr after the end of the final treatment, and the number of surviving tumour clonogens determined in culture. Values are mean ± SEM for groups of 6 mice. * indicates values that were significantly greater ($p<0.05$) than for radiation alone by ANOVA/Dunnett's.

6. **The results show** that SN 29751 is less toxic than TPZ to mice, on either a molar or mass basis, and that at doses providing equivalent toxicity SN 29751 has activity against hypoxic cells which is at least equal to TPZ and in most tumour models clearly superior to TPZ.

The toxicity of TPZ to CD-1 nude mice is shown as a function of dose in Table 1; from these data the MTD is estimated as 178 µmol/kg (31.7 mg/kg) for a single IP dose, and 56.2 µmol/kg (10 mg/kg/dose) for the BID1-4 schedule. Similarly, the toxicity of SN 29751 is shown as a function of dose in Table 2; the MTD estimated from these data was 1000 µmol/kg (371 mg/kg) for a single IP dose and 421 µmol/kg (156 mg/kg/dose) for the BID1-4 schedule.

Table 1: toxicity of TPZ in male CD-1 nude mice.

Schedule	Dose (mg/kg/dose)	Mortality	Body weight loss at nadir (% of pre-treatment)	
			Mean	SEM
Single dose	17.8	0/3	4.5	1.4
Single dose	23.7	0/6	8.2	1.8
Single dose	31.7 (MTD)	0/9	4.6	1.2
Single dose	42.2	18/18	15.3	0.8
Single dose	56.3	3/3	18.1	2.2
BID 1-4	10.0 (MTD)	0/8	5.0	0.9
BID 1-4	13.4	3/12	11.3	1.0

Table 2: Toxicity of SN 29751 in male CD-1 nude mice

Schedule	Dose (mg/kg/dose)	Mortality	Body weight loss at nadir (% of pre-treatment)	
			Mean	SEM
Single dose	370.8 (MTD)	0/6	-0.1	1.4
Single dose	493.2	3/6	10.1	3.7
BID 1-4	87.9	0/3	1.5	1.0
BID 1-4	117.2	0/3	0.6	1.8
BID 1-4	156.1 (MTD)	0/9	11.9	1.5
BID 1-4	208.4	2/3	-1.0	0.8

The activity of TPZ and SN 29751 in combination with single dose or fractionated radiation against human tumour xenografts is compared in Figures 1-6, using clonogenic assays to measure surviving cells 18 hr after the end of treatment. In the single dose experiments, a large dose of ionising radiation is used to kill aerobic cells, allowing evaluation of the ability of the compounds to kill the hypoxic (radioresistant) surviving cells. In Figure 1, both compounds have significant activity against SiHa tumours when administered either before or after irradiation. Their activity when given after irradiation demonstrates that the compounds are acting as hypoxic cytotoxins rather than classical radiosensitizers (which interact with radiation-induced free radicals and must therefore be present within milliseconds of irradiation). Notably, SN 29751 was clearly more active than TPZ in this assay. Figure 2 confirms the activity of both compounds post-irradiation, and shows that SN 29751 is more active than TPZ against all three tumour models evaluated.

Figure 3 shows the results of initial screening experiments with fractionated radiation, which demonstrate that SN 29751 has greater activity than TPZ when administered 30 min before each dose of radiation against H460 and H1299 tumours. Figure 4 shows the time course of interaction with radiation, against SiHa tumours. As for the single dose schedule, both compounds were active before as well as after irradiation, but SN 29751 showed higher activity. Figure 5 shows a dose-response experiment in which the compounds were administered 30 min before each dose of radiation against SiHa tumours. The highest dose of TPZ tested was its MTD on this schedule, while for SN

29751 the highest dose was 75% of MTD, yet SN 29751 clearly provided greater tumour cell killing additional to radiation at the highest doses. A similar dose-response experiment was performed with H460 tumours (Figure 6), demonstrating that the highest dose of SN 29751 tested (56% of MTD) was as active as TPZ at its MTD.

I declare that all statements herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

William R Wilson

8 December 2009

Date

Figure 1

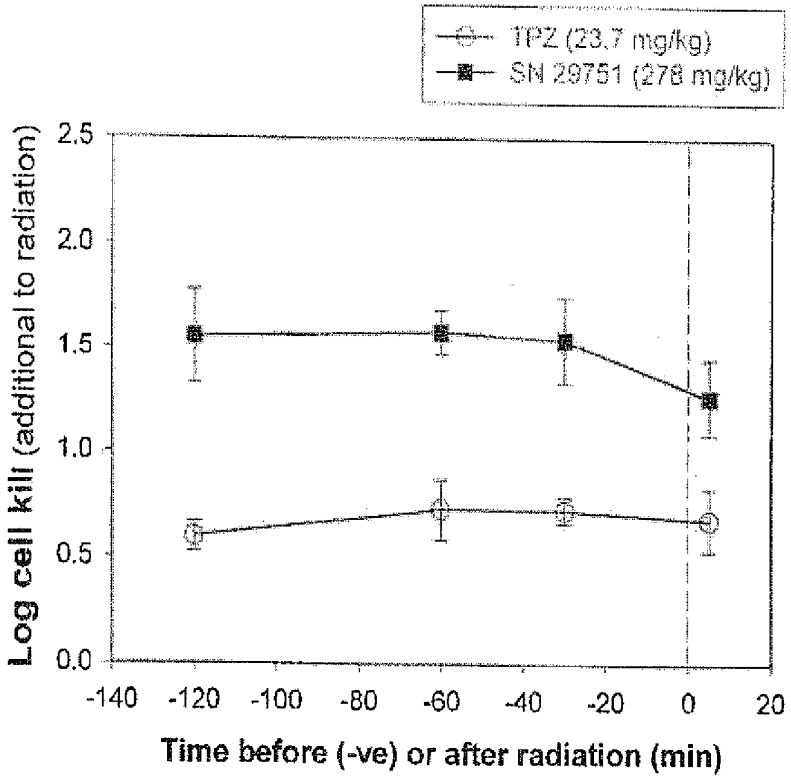

TPZ (23.7 mg/kg)
SN 29751 (278 mg/kg)
Log cell kill (additional to radiation)
2.5
2.0
1.5
1.0
0.5
0.0
-140
-120
-100
-80
-60
-40
-20
0
20
Time before (-ve) or after radiation (min)

Figure 2

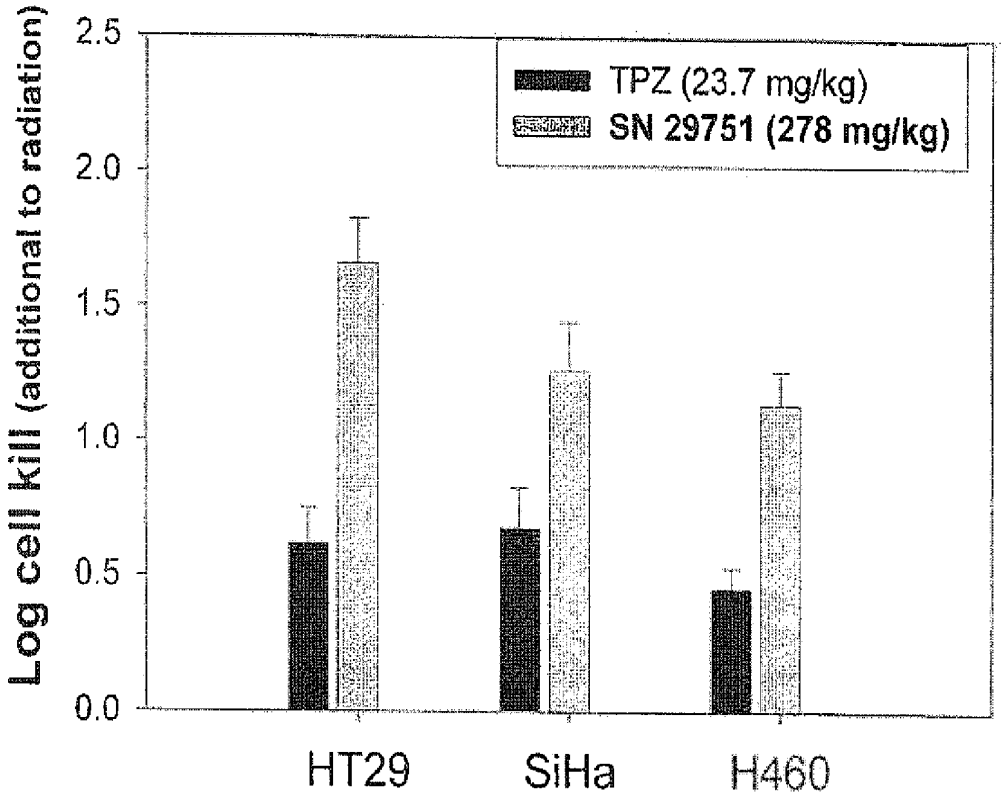

Log cell kill (additional to radiation)
2.5
2.0
1.5
1.0
0.5
0.0
TPZ (23.7 mg/kg)
SN 29751 (278 mg/kg)
HT29
SiHa
H460

Figure 3

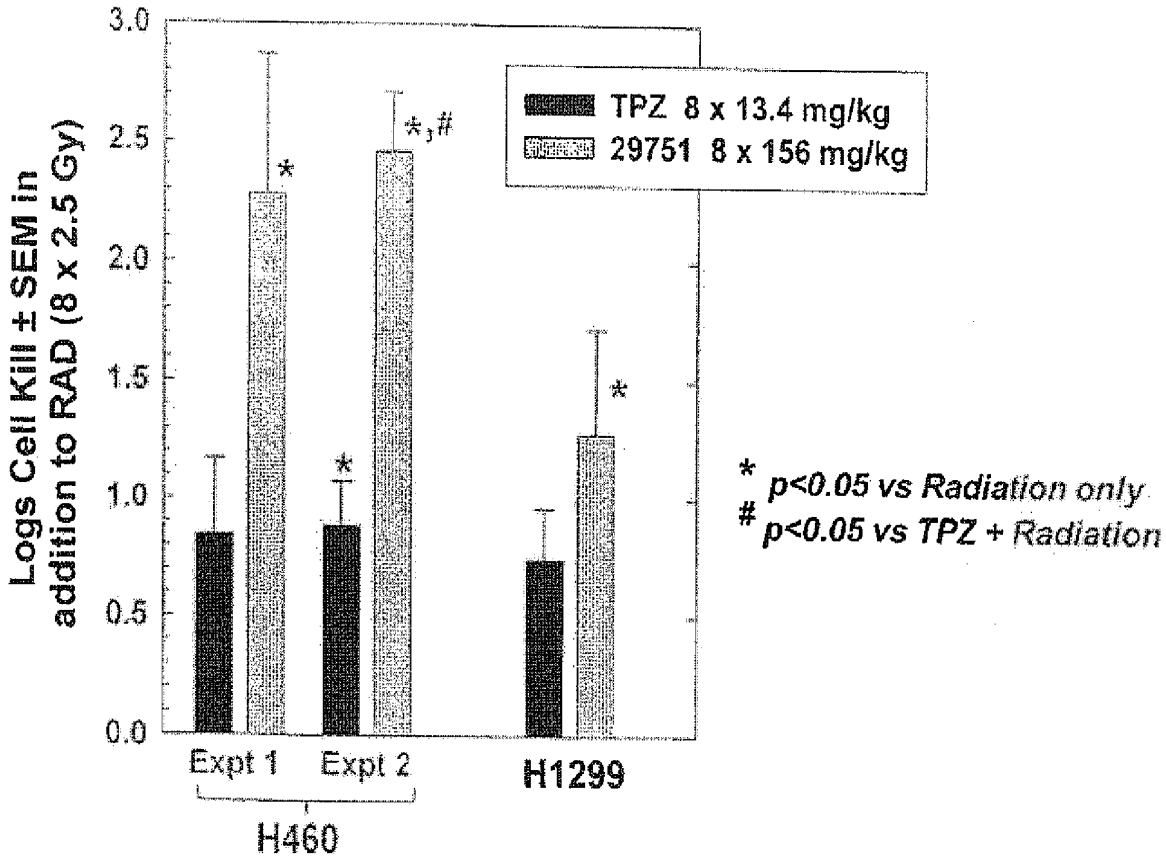

Logs Cell Kill ± SEM in addition to RAD (8 x 2.5 Gy)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TPZ 8 x 13.4 mg/kg
29751 8 x 156 mg/kg
*
*
*,#
*
Expt 1
Expt 2
H460
H1299
* p<0.05 vs Radiation only
# p<0.05 vs TPZ + Radiation

Figure 4

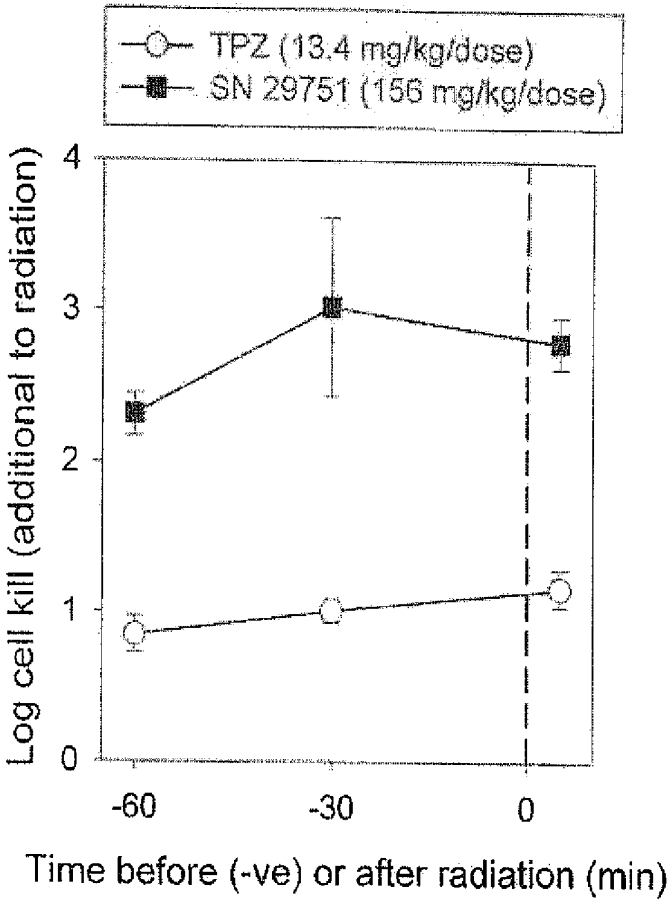

TPZ (13.4 mg/kg/dose)
SN 29751 (156 mg/kg/dose)
Log cell kill (additional to radiation)
0
1
2
3
4
-60
-30
0
Time before (-ve) or after radiation (min)

Figure 5

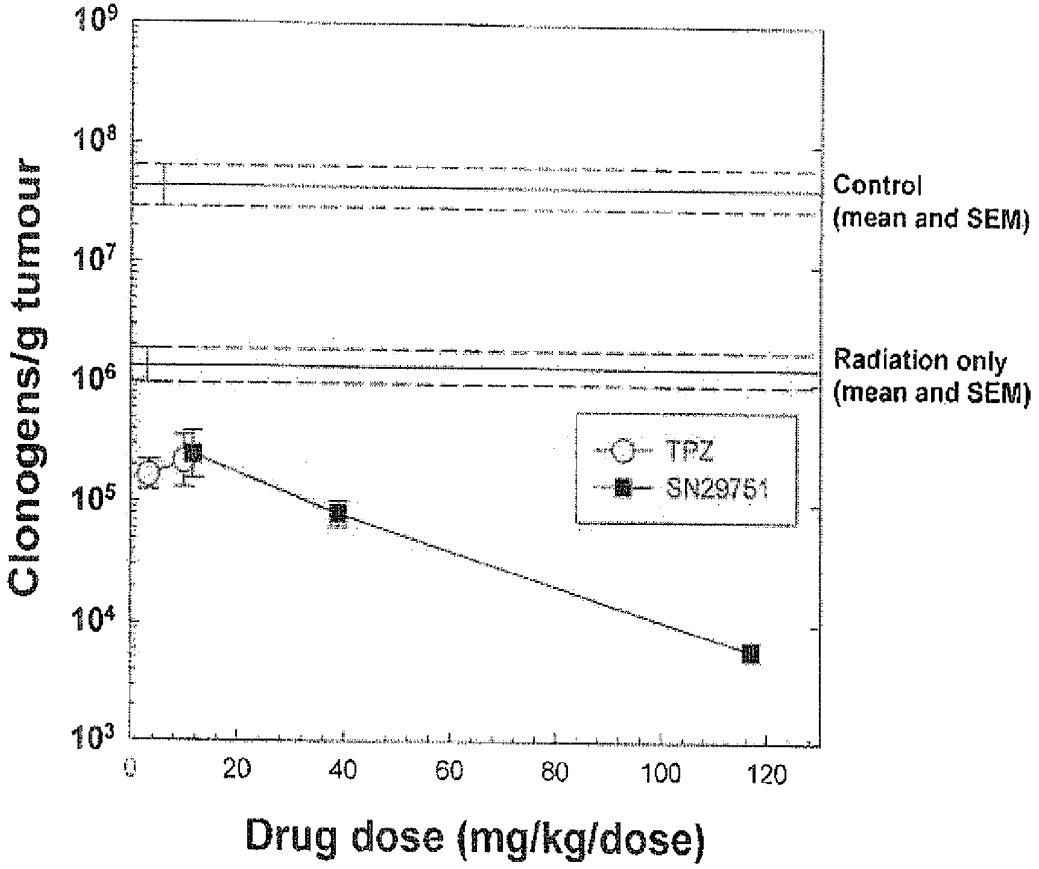
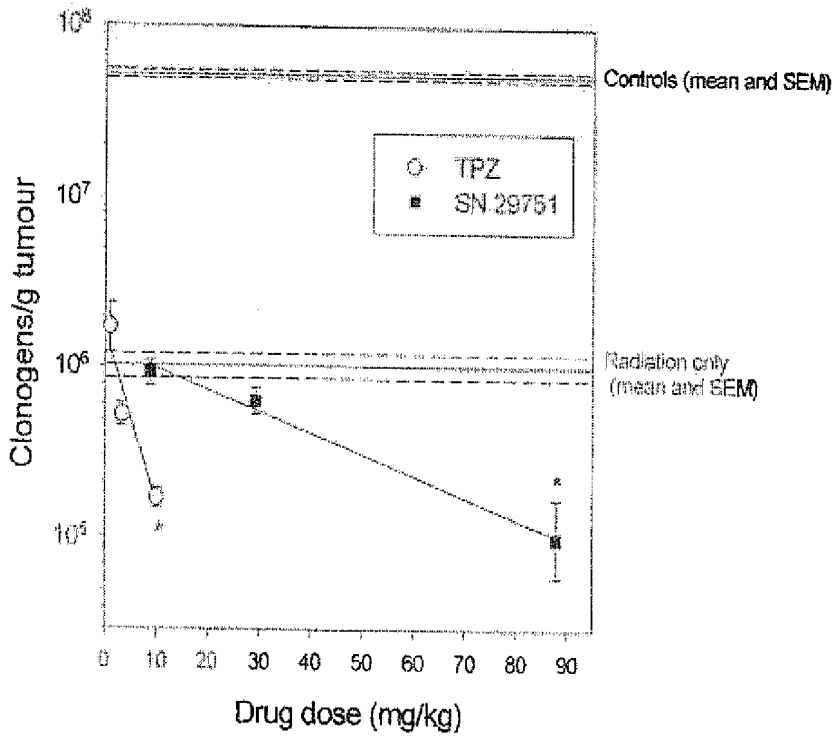

Clonogens/g tumour
10^9
10^8
10^7
10^6
10^5
10^4
10^3
0
20
40
60
80
100
120
Drug dose (mg/kg/dose)
TPZ
SN29751
Control
(mean and SEM)
Radiation only
(mean and SEM)

Figure 6


Controls (mean and SEM)
TPZ
SN 29751
Radiation only
(mean and SEM)
Clonogens/g tumour
Drug dose (mg/kg)
0
10
20
30
40
50
60
70
80
90